

Comparison of γ -glutamyl hydrolase (conjugase; EC 3.4.22.12) and amylase treatment procedures in the microbiological assay for food folates

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1. It has been suggested that deconjugation of food folates with pig kidney, compared with chicken pancreas, may increase measurable folate by approximately 50% (Phillips & Wright, 1983). Therefore deconjugation with conjugases from these two enzyme sources was optimized and compared. Folate was measured microbiologically with *Lactobacillus casei* (ATCC 7469) as the test organism at pH 5.6.
2. Treatment for 6 h with 200 mg pig kidney/200 mg sample was compared with the conventional assay employing overnight incubation with 20 mg chicken pancreas/5 g sample. Comparison of the deconjugation systems showed chicken pancreas to be superior for peas (*Pisum sativum*) and beans (*Phaseolus vulgaris*), while there was no difference for potatoes.
3. γ -Glutamyl hydrolase (conjugase; EC 3.4.22.12) treatment for 2 and 20 h with pig kidney and chicken pancreas was optimized for raw potatoes and green frozen peas. Treatments with pig kidney were conducted at pH 4.6, which is optimal, and at pH 5.2. There was no significant difference between 2 and 20 h treatments at pH 5.2. Treatments with chicken pancreas were conducted at pH 6.1. Treatment for 2 h was preferred as it resulted in significantly higher measurable folate activity in peas and potatoes, and because overnight treatment can be influenced by microbial production of folate.
4. With optimal treatment conditions the source of enzymes did not significantly influence measurable folate activity. Chicken pancreas is the traditional source of conjugase in Scandinavia and was preferred for deconjugation.
5. Chicken pancreas, 20 and 60 mg, was used for deconjugation of sixteen different food samples, each containing approximately 200 ng folate. Chicken pancreas at 60 mg/sample gave significantly higher results. Further addition of enzyme did not increase the folate values.
6. A combined amylase treatment using heat-resistant α -amylase (EC 3.2.1.1) during extraction to ensure the gelatinization of the sample, and glucoamylase (EC 3.2.1.3), along with the incubation with chicken pancreas to complete the digestion, was shown to give a small but significant increase in folate values of starch-containing samples.
7. Folate results using the recommended procedure are given for raw potatoes, wheat bran, rolled oats, wheat flour and dark rye bread.
8. Chicken pancreas was shown to contain equally high amounts of amylases as did the α -amylase and amyloglucosidase sources. This finding may explain the relatively small benefit of a specific amylase addition.

Folate is the generic name for a large number of chemical derivatives of pteroylmonoglutamic acid (folic acid), which belong to the vitamin-B group. Folate is necessary for the synthesis of DNA, and the first sign of depletion is usually megaloblastic anaemia. The principal folates in food are polyglutamates with the pteroyl moiety fully reduced and with 1-carbon substitutions at one or more of the nitrogen atoms 5 and 10.

The World Health Organization (1972) and (US) National Research Council (1980) recommend an intake of 400 μg folate/d for adults. The National Research Council assumes that 100-200 μg folic acid/d are needed to maintain tissue reserves and that 25-50% of dietary folates are nutritionally available. These assumptions have been questioned, as the scientific evidence underlying the stipulation of the minimal requirements is quite weak (Bates *et al.* 1982; Anon., 1985). Herbert (1987) recently reviewed the folate requirements and concluded by recommending dietary intakes of 3 μg folate/kg body-weight per d. It may be noted, however, that the recommended dietary allowance (RDA) values are usually designed to be well above the absolute minimum requirements. This ideal

seems to be fulfilled in the case of folate. A number of investigations have shown that the actual daily intakes of large parts of the populations of the world are below the RDA values, although megaloblastic anaemia is certainly not that widespread (Hoppner *et al.* 1972; Jägerstad & Westesson, 1979; Bates *et al.* 1980; Rosenberg *et al.* 1982; Rogozinski *et al.* 1983).

In order to establish a consensus on the folate situation including RDA values, clinical manifestations of the depletion and calculations of daily intakes, there is a need to look critically at the RDA, symptoms of depletion and food table values. The present paper deals with the microbiological analysis of folate providing the values for food tables.

The analysis of folates in food is commonly performed microbiologically using as the test organism *Lactobacillus casei* (ATCC 7469) which can utilize not only mono-, di- and triglutamates but also to some extent polyglutamates with more than three glutamates (Tamura *et al.* 1972). It can utilize most derivatives of folic acid including its methyl and formyl derivatives (Krumdieck *et al.* 1983).

In order to improve the analysis of polyglutamates with three or more glutamic acid residues, food extracts are treated with γ -glutamyl hydrolase (conjugase; EC 3.4.22.12). Polyglutamates are deconjugated into diglutamates with enzymes from chicken pancreas (Käferstein & Jaenicke, 1972; Leichter *et al.* 1979), or into monoglutamates with enzymes from pig kidney (Bird & McGlohon, 1972).

In the past most food tables have included two values for folates: total folates as well as 'free folate'. The latter is defined as the part of folates measurable with *L. casei* without addition of any foreign conjugase system. It was assumed that the absorption of free folates is better than that of other folates in the human digestive tract.

Three problems are associated with the distinction between free folate and total folate. First, in the human digestive tract folates are deconjugated into monoglutamates before absorption (Rosenberg, 1976). The term free folate, as defined previously, is misleading as it encompasses folates with one, two, three or even more glutamates. Second, many foods contain conjugases which may act in food extracts on homogenization. Traditional assays not taking this deconjugation into account may overestimate the amount of free folate in many foods (Malin, 1976; Bender & Nik-Daud, 1983; Jakobsen *et al.* 1985). Third, humans, young and elderly, probably absorb polyglutamates just as well as they absorb monoglutamates (50–60%) (Tamura *et al.* 1978; Bailey *et al.* 1984), indicating that insufficient deconjugation, except for some rare diseases, does not limit absorption of folates. In agreement with this, Rosenberg (1976) has suggested that the limiting step in digestion of folates is the absorption of monoglutamates. Thus low body levels of folates are usually not the result of insufficient deconjugation of polyglutamates. Depletion, more often, is a result of insufficient diets due to social causes (Wagner *et al.* 1981; Rosenberg *et al.* 1982; Rogozinski *et al.* 1983).

For these reasons we have decided to stop compilation of 'free folate' in Danish food tables. On the other hand there is still a scientific need to investigate which forms of folates actually occur in different organisms and organs in order to understand the regulatory mechanisms in which folates are involved. Such investigations must employ exact methods to distinguish between the different folates.

In the analysis of folate it is intended to measure the total amount of folates. Recently Phillips and Wright's group has claimed that replacement of chicken pancreas conjugase with a pig-kidney preparation would result in substantially higher levels of folate (approximately 50% higher) (Bates *et al.* 1982; Phillips *et al.* 1982, Phillips & Wright, 1983). Their samples included garden peas (*Pisum sativum*), potatoes, runner beans (*Phaseolus coccineus*), brussels sprouts (*Brassica oleracea* var. *gemmifera*) and Marmite®. Another comparison between these enzyme sources has been performed by Kirsch & Chen

(1984) using spinach (*Spinacea oleracea*). They did not find any significant difference between these enzyme sources. They remarked that the discrepancy with the results of Phillips & Wright (1983) might be due to different procedures for preparation of conjugase solutions, incubation time and substrate (food samples).

Cerná & Kás (1983) have published results which suggest that treatment with α -amylase (*EC* 3.2.1.1) and in one case also glucan 1,4- α -glucosidase (amyloglucosidase; *EC* 3.2.1.3) might be necessary to release folates bound to starch by physical adsorption. Employing heat-resistant α -amylase, M. Jägerstad (personal communication) has confirmed the benefit of amylase treatment.

In the present investigation deconjugation with the two sources of conjugases, pig kidney and chicken pancreas, has been optimized for two incubation periods and a fixed amount of sample-folate from two samples: green frozen peas and potatoes. The best treatments were compared and chicken pancreas was chosen for a final optimization experiment comparing two enzyme levels for sixteen different foods. The folate results with and without a mixed amylase treatment, combining the effects of α -amylase and amyloglucosidase, were compared.

MATERIALS AND METHODS

Extraction of samples

Samples were autoclaved for 5 min at 120° with 100 ml phosphate buffer (0.2 M containing 5 g ascorbic acid/l, pH 6.1). Cooled extracts were homogenized if necessary, filtered, diluted with buffer, and then frozen at -20° in 1-7 ml portions containing about 200 ng folate.

Preparation of conjugase sources

A solution containing 46.4 mg pig kidney/ml was prepared according to a method adopted from Phillips & Wright (1983). Dried pig kidney (Sigma Chemical Co., Poole, Dorset; 13 g) was homogenized with 200 ml L-cysteine hydrochloride (10 g/l) which had been adjusted to pH 4.6 with sodium hydroxide. The homogenate was incubated for 2 h at 37° and then centrifuged at 2000 g for 10 min. To the supernatant fraction was added 40 ml activated charcoal-dextran mixture (1 g Dextran T 40 (Pharmacia) and 10 g Darco G-60 activated charcoal (Fisher) in 100 ml water). After 30 min at room temperature this was centrifuged at 2000 g for 10 min. The supernatant fraction was treated once more with 40 ml activated charcoal-dextran mixture, centrifuged at 40000 g for 30 min and stored at -20°.

With minor modifications chicken pancreas was treated in the same way. Dried chicken pancreas (Difco; Expt 2, 3.2 g; Expts 3 and 4, 6.4 g) was homogenized with 200 ml 0.2 M-phosphate buffer (pH 6.1) and pH was adjusted to 4.6 before each treatment with 40 ml activated charcoal-dextran mixture. Chicken pancreas was treated three times with activated charcoal-dextran mixture, adjusted to pH 6.1 and stored at -20°.

Deconjugation of sample extracts

Expt 1. Deconjugation with chicken pancreas was performed according to the conventional procedure in the National Food Agency of Denmark (Nordic Committee on Food Analysis, 1985): a 5 g food sample was extracted in 100 ml buffer as described previously. Dried chicken pancreas (Difco; 20 mg) was added and the mixture was incubated under toluene overnight at 37°.

Deconjugation with pig kidney was based on the assay of Phillips & Wright (1983). A 100 mg pig kidney/ml solution was treated once by the activated charcoal-dextran

procedure described previously. Of this solution 2 ml was mixed with 1 ml of an extract containing 0.4 g (potatoes), 0.2 g (peas) or 0.12 g (dried white beans (*Phaseolus vulgaris*)) substrate, each containing 100–200 ng folate, and 7 ml of a 20 g ascorbic acid/l solution (pH 5.6). This mixture was incubated for 6 h at 37°.

With both enzyme sources enzyme blanks were run for each assay and a correction was made.

Expts 2 and 3. A sample extract containing 200–250 ng folate and the appropriate amount of enzyme were made up to 10 ml with phosphate buffer, pH 4.6 (pig kidney) or 6.1 (chicken pancreas) and incubated for 2 or 20 h at 37°. Toluene was added to samples incubated overnight. After deconjugation, samples were steamed and diluted to an estimated 0.9 ng folate/l with 5 g ascorbic acid/l solution (pH 5.6) or 0.2 M-phosphate buffer (pH 6.1) with 5 g ascorbic acid/l. The buffer is now preferred since it ensures the same pH value (pH 6.0) in all samples and standard solutions (Phillips & Wright, 1982).

Amylase treatment

In Expts 3 and 4, samples known to contain storage carbohydrates were treated with amylases: by extraction, 200 μ l α -amylase (Termamyl 300 L type B; Novo)/5 g dry matter was included before autoclaving. Amyloglucosidase (Sigma A9268, treated twice with activated charcoal-dextran mixture; 200 μ l) was added at the same time as the chicken pancreas.

Standard solution

Pteroylmonoglutamic acid (folic acid) was an International Chemical Reference Substance from the World Health Organization (Solna, Sweden). An amount equivalent to 10.000 mg water-free folic acid was dissolved in 90 ml water with one to two drops of 1 M-sodium hydroxide, the pH was adjusted to 7–8 (0.1 M-hydrochloric acid) and water was added to 100 ml. This stock solution was used for 14–30 d. The concentration of a new solution was verified spectrophotometrically at 281 nm.

Microbiological assay

L. casei (ATCC 7469) was maintained in agar. Liquid culture medium was prepared by adding 25 ng folic acid/ml to Dano Folic Acid Casei Medium, prepared according to the manufacturer. Culture media (5 ml) were inoculated from agar stabs and incubated overnight at 37°. Cultures were then centrifuged twice and finally redissolved in 100 ml saline (9 g sodium chloride/l). One drop per tube (approximately 0.05 ml) of this suspension was used for inoculation.

Double-strength basal medium was prepared from Dano Folic Acid Casei Medium as described by the manufacturer. Ascorbic acid (5 g/l) was added just before use and pH was adjusted to 5.6.

To eight tubes containing 2.5 ml double-strength medium were added 0.5, 1.0, 1.5 and 2.0 ml, in duplicate, of either standard folic acid solution (0.6 ng/ml or 1.2 ng/ml in each assay) or sample extract. Four blanks were included in each assay. Volumes were made up to 5 ml with 5 g ascorbic acid/l solution (pH 5.6) or 0.25 M-phosphate buffer (pH 6.1) with 5 g ascorbic acid/l as described previously. Tubes were capped, autoclaved for 10 min at 110°, inoculated, and incubated for 18–22 h at 37°. Turbidity was measured at 650 nm and results calculated by quadratic regression analysis.

Statistics

Values were analysed by Student's *t* test and analysis of variance.

Table 1. *Expt 1**. Contents of folate (ng/g) after deconjugation with chicken pancreas (20 mg/5 g sample, incubated overnight), or pig kidney (200 mg/120–400 mg sample, incubated for 6 h)

(Mean values and standard deviations for six determinations)

	Potatoes		White beans (<i>Phaseolus vulgaris</i>)		Green peas (<i>Pisum sativum</i>)	
	Mean	SD	Mean	SD	Mean	SD
Chicken pancreas	292	38	2070	346	1120	67
Pig kidney	293	162	1420	135	803	212

* For details, see pp. 263–264.

RESULTS

Expt 1

Initially the conventional deconjugation procedure employing uncleaned, dried chicken pancreas was compared with the pig-kidney deconjugation of Phillips & Wright (1983) for raw potatoes, dried white beans and green frozen peas homogenized before or after autoclaving.

As measureable folate activity did not vary significantly whether samples were homogenized before or after autoclaving, results were pooled (Table 1). For potatoes there was no significant difference between the two treatments, but the standard errors with pig kidney were large due to an enzyme-blank correction varying from 52 to 90 % of the results. When potatoes were omitted an analysis of variance showed that deconjugation with chicken pancreas yielded significantly higher results than pig kidney ($P < 0.001$).

Expt 2. Optimization of deconjugation systems

Enzymes were treated with activated charcoal which binds free folates. As delivered from the manufacturer, dried powder of pig kidney and chicken pancreas contained 5 and 6 ng folate/mg respectively. Following the described purification the 46.4 mg pig kidney/ml solution contained about 0.5 ng folate/ml or 0.01 ng/mg. Depending on enzyme concentration (shown in mg/ml in parentheses) the different solutions of chicken pancreas contained < 0.15 (10), 0.3 (30) and 0.9 (45) ng/ml, i.e. < 0.015, 0.01 and 0.02 ng folate/mg. In all cases the content of folate is reduced by a factor of 500, approximately.

Conjugase activity was determined on dried bakers yeast. The activity loss due to the purification procedure for chicken pancreas was about 50 %. On a weight basis pig kidney had about 10 % the activity of chicken pancreas on dried yeast.

Typical dose–response curves from the optimization experiments are shown in Fig 1. Results are represented in Table 2. For each source of enzyme, incubation time and sample, dose–response curves were the same basic shape with a plateau, indicating that enzyme doses above a certain level will not release further significant amounts of measureable folate. Enzyme doses below this threshold level were discarded before the statistical evaluation of treatments.

The content of folate in the extracts treated with enzyme was of the same order of magnitude: 230 ng for potatoes and 340 ng for peas, and there was good agreement between enzyme demands of the two samples. When incubation was prolonged from 2 to 20 h less enzyme was needed, especially in the case of pig kidney. With chicken pancreas

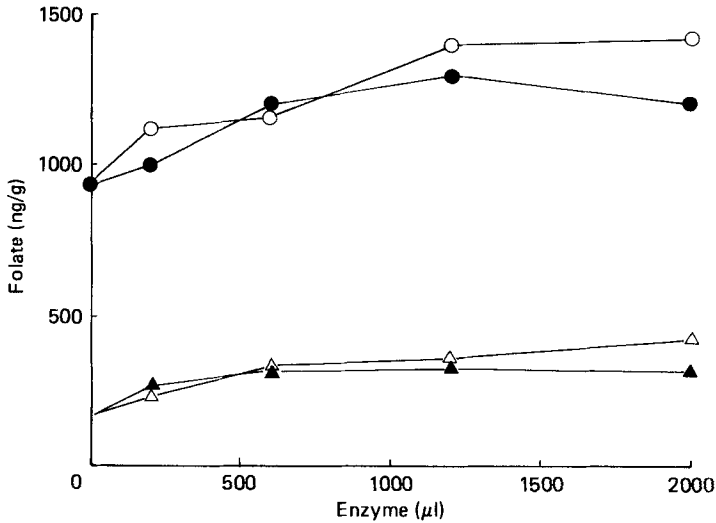


Fig. 1. Optimization of deconjugation with 10 ng chicken pancreas/ml for peas (*Pisum sativum*) (○—○) and potatoes (△—△) and 46.4 mg pig kidney/ml (pH 5.2) for peas (●—●) and potatoes (▲—▲). Incubation period 2 h.

the folate level decreased 20% with both samples when incubation was prolonged from 2 to 20 h. These differences were highly significant, while the 4 and 8% differences observed with pig kidney were not significant. Further, cereal samples, like wheat flour and rolled oats, when incubated overnight under toluene, gave large problems with microbial growth producing folate, leading to unexpectedly high results and variations up to 400% between measurements. Thus for both enzyme sources the shorter incubation period was preferred.

With pig kidney most experiments were performed at pH 5.2, which is the immediate pH when extracts (pH 6.1) were mixed with buffer (pH 4.6). In comparison with experiments conducted at pH 5.2, incubation for 2 h at pH 4.6 yielded significantly higher folate values with potatoes and equal values with peas. Consequently it can be concluded that deconjugation with pig kidney should be performed at pH 4.6.

Finally the two sources of enzymes were compared. The results from 2 h treatment with chicken pancreas were significantly higher than those obtained with pig kidney at pH 5.2. When compared with pig kidney at pH 4.6, chicken pancreas values were not significantly different. It was concluded that pig kidney does not yield higher amounts of measureable folate activity with these samples than chicken pancreas. Therefore pig kidney was discarded from the final optimization experiment.

Expt 3. Further optimization of chicken pancreas treatment

From Expt 2, 2000 μl chicken pancreas solution was chosen as an appropriate amount of enzyme for testing a broad panel of foods. This level and three times this level (20 and 60 mg) was compared for deconjugation of sixteen different kinds of food representing the most important contributions to the Danish folate intake. Results are shown in Table 3.

Measurements of individual samples were repeated when the difference between enzyme treatments exceeded 20% (laboratory 99% confidence level for double determination). No difference was significant on double determinations, but overall, treatment with 60 mg chicken pancreas yielded significantly higher results than 20 mg (*t* test, $P < 0.01$). The average difference was 14%. Further addition (150 mg) of chicken pancreas to those two

Table 2. Expt 2†. Optimization of deconjugation with chicken pancreas (CP) and pig kidney (PK)

(Values are measurable folate activity (ng/g sample) with 95% confidence limits (CL). Plateau levels were computed as the mean of the higher enzyme levels, excluding those that were judged to be on the ascending part of the graph)

Incubation period (h)...	20									
	CP		PK		CP		PK		PK	
Enzyme source... pH of deconjugation...	6.1	6.1	5.2	5.2	6.1	6.1	4.6	4.6	5.2	5.2
Enzyme volume (μ l)	Mean	CL	Mean	CL	Mean	CL	Mean	CL	Mean	CL
Potatoes										
0	170†	18	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	240†	78
60	—	—	—	—	—	—	—	—	300	76
200	230†	51	270†	95	—	—	—	—	300	130
600	320†	66	320	10	340	180	350	70	310	100
1200	350	53	330	75	410	100	280	84	290	110
2000	420	44	320	82	—	—	—	—	—	—
Plateau level	—	—	326 ^{a,b,*}	23	373 ^{a,b,*}	68	309 ^c	34	300 ^c	34
Peas (<i>Pisum sativum</i>)										
0	918†	53	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—
60	—	—	—	—	—	—	—	—	1100†	190
200	1100†	170	1000†	190	—	—	—	—	1200	60
600	1200†	270	1200	170	1400	400	980†	210	1200	140
1200	1400	590	1300	370	1200	210	1100	220	1200	190
2000	1400	385	1200	70	—	—	—	—	1200	230
Plateau level	1402 ^d	190	1229 ^{d,e,*}	99	1238 ^e	138	1129 ^{e,*}	99	1178 ^e	38

a-e Plateau levels with different superscript letters were significantly different ($P < 0.05$).

* $0.1 > P > 0.05$.

† For details, see p. 264.

‡ Values excluded from computation of plateau levels.

Table 3. *Expt 3**. Comparison of folate values after deconjugation with 20 and 60 mg chicken pancreas (CP) for sixteen foods

(Values are for single determinations)

Sample	20 mg CP (ng/g)	60 mg CP	
		ng/g	% 20 mg CP value
Pork, lean, raw	17	18	107.2
Liver, pig, raw†	5400	6100	113.0
Cod, fillet, raw	110	120	106.4
Milk, 35 g fat/kg, homogenized	69	75	108.6
Cheese, firm, 450 g fat/kg dry matter	580	630	109.4
Egg, chicken, yolk, raw	1300	1300	99.2
Egg, white, raw	27	30	109.3
Rye bread, dark†	420	450	108.4
Wheat flour, enriched†	150	210	134.9
Oats, rolled†	540	590	109.7
Cabbage, white (<i>Brassica oleracea</i> var. <i>capitata</i>), raw†	210	250	115.3
Potatoes, raw†	420	440	105.5
Carrot, raw†	360	380	105.3
Spinach (<i>Spinacea oleracea</i>), chopped, frozen†	1100	1500	146.7
Peas (<i>Pisum sativum</i>), green, frozen†	1500	1600	108.3
Orange, raw	520	740	141.1
Mean of sixteen samples			114
SD			14

* For details, see p. 264.

† Treated with amylases.

samples showing the greatest difference (oranges and spinach) yielded 86 and 87% respectively of the results obtained with 60 mg. Therefore 60 mg was assumed to be the optimal enzyme level for treating food extracts containing about 200 ng folic acid.

Expt 4. Amylase treatment

Conditions for treating samples with α -amylase and amyloglucosidase were established from experiments with rolled oats and wheat flour. With increasing amounts of α -amylase a small (10%, insignificant) increase in folate values was observed for rolled oats, but not for wheat flour. Addition of amyloglucosidase to α -amylase-treated samples released another 10% of folate from rolled oats (difference significant in comparison with untreated ($P < 0.002$) or α -amylase-treated ($P < 0.05$) samples). From these experiments it was decided to employ a combination of α -amylase and amyloglucosidase treatment for all cereals and other starch-containing samples, as described previously. This treatment was compared with no addition of amylases with four samples of each of five different foods (Table 4). Overall treatment with amylases increased the amount of folate by 9%. A two-way analysis of variance showed this difference to be significant when results were expressed as a percentage of the sample mean ($P < 0.02$).

The amylase contents of chicken pancreas were determined by the Novo Co. Copenhagen. In chicken-pancreas powder, 266 kNovo amylase units (Novo AF9) and 243 Novo amyloglucosidase units (Novo AF22) were detected.

Table 4. *Expt 4**. Comparison of folate contents on treatment with and without α -amylase (EC 3.2.1.1) and glucan 1,4- α -glucosidase (amylglucosidase; EC 3.2.1.3)

(Values are for single determinations)

Sample	Without amylases (ng/g)	With amylases	
		ng/g	% without amylases value
Wheat bran	1300	1400	107
	1300	1300	104
	1500	1000	70.6
	1500	1800	124
Oats, rolled	370	400	108
	450	440	97.8
	360	580	162
	460	590	129
Potatoes, raw	350	270	78.0
	350	400	115
	400	440	109
	340	410	119
Rye bread, dark	600	550	92.0
	480	540	112
	380	480	126
	430	450	104
Wheat flour	130	170	133
	170	180	104
	330	270	83.7
	190	200	108
Mean	—	—	109.3
SD	—	—	20.6

* For details, see p. 264.

DISCUSSION

Optimization of deconjugations

In the present investigation the amount of deconjugation enzyme was adjusted to the expected folate content of the samples. Thus it was assumed that the deconjugase demand is determined mainly by the content of folates. This assumption was found to be justified in the case of potatoes and green peas.

Incubation for 2 h is recommended in the present report. The period of 2 h was chosen for the experiments as a compromise between an effort to keep enzyme demands low, due to the problems associated with enzyme blank corrections, and an effort to keep incubation adequately short so that samples can be extracted and incubated within one working-day. Bacterial contamination by overnight incubation has also been reported by Jägerstad *et al.* (1975). In contrast to our observations, they found that the problem could be controlled by incubation under toluene.

The observation of a declining folate content, when incubation with chicken pancreas was prolonged from 2 to 20 h, can be explained by degradation of folates during incubation (Chen & Cooper, 1979).

The amount of conjugase enzyme which was found to be optimal in the present investigation can be compared with the levels reported in other optimization experiments and with the levels recommended by different authors. Kirsch & Chen (1984) found that a 2 h incubation period was enough to complete deconjugation of 0.5 μg folate in spinach with 1.5 mg chicken pancreas, i.e. 0.5 mg chicken pancreas/200 ng sample-folate. The 0.5 mg chicken pancreas should be compared with the 20 mg level used in Expt 2 of the present study. The disagreement might be due to the different substrates (samples).

With a fixed incubation period of 6 h, Phillips & Wright (1983) found that 100 mg pig kidney and 20 mg chicken pancreas were enough to deconjugate 34 ng folate in frozen green peas. These values are of the same order of magnitude as those found in Expt 2.

From our experiments we recommend deconjugation for 2 h with 60 mg chicken pancreas/200 ng folate. This amount of chicken pancreas is in good agreement with the recommendation of Bell (1974) (25 mg chicken pancreas/100–500 ng folate, 16 h) and somewhat more than our conventional assay (20 mg chicken pancreas/2000–8000 ng folate, overnight). From measurement of enzyme activity (Chen *et al.* 1983), it can be seen that 60 mg chicken pancreas should be capable of deconjugating about 10 mg synthetic pteroyltriglutamate during the 2 h incubation period. Thus the optimal application of chicken pancreas will give a large surplus of conjugase, about 10^5 times the theoretically sufficient amount.

Although conjugase inhibitors are known (Tamura & Stokstad, 1973) the requirement for such a large surplus is surprising. One explanation might be that factors (enzymes) other than the conjugase are of importance.

The benefit of treating starch-containing samples with α -amylase and amyloglucosidase was marginal, but significant. An explanation of the small increase in folate contents might be the presence of amylases in chicken pancreas. The amounts of amylases present in chicken pancreas per g sample are of the same order of magnitude as the amounts of added α -amylase and amyloglucosidase. Thus the amylase content might be an important factor in the determination of the optimal amount of chicken pancreas.

In accordance with the findings of Kirsch & Chen (1984), but contrary to those of Phillips & Wright (1983), deconjugation with pig kidney compared with chicken pancreas did not give higher results of measureable folate. Phillips & Wright (1983) reported 1.34 μg folate/g using pig kidney and 0.80 μg folate/g using chicken pancreas for garden peas and 420 ng folate/g using pig kidney for potatoes. The corresponding values from the present investigation are 1.402 $\mu\text{g/g}$, 1.238 μg and 386 ng/g respectively. Thus there is a good agreement between the pig-kidney results of Phillips & Wright (1983) and the present results, while the results of Phillips & Wright (1983) with chicken pancreas for garden peas are much lower than the present results.

It can be concluded that, although pig kidney may be preferred for some studies because the end-products of deconjugation are monoglutamates, it is not in general a better source of conjugase than chicken pancreas. For the compilation of food tables both enzyme sources may be used, but it is important to ensure that the deconjugation procedure is optimized. Generally short-term incubation, that is 1–4 h, should be preferred.

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